

Rapid isothermal detection assay: a probe amplification method for the detection of nucleic acids

Wenjuan Gao, Xiang Li, Lingwen Zeng, Tao Peng*

Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510663, China

Abstract

Simple, accurate, and stable diagnostic tests are essential to control viral infectious diseases such as avian influenza virus. The current technologies are often inaccessible to people who need them, mainly because of the specialized equipment and the need for highly trained technologists. Here, we describe a rapid isothermal nucleic acid detection assay (RIDA) that can be used to detect both DNA and RNA targets. Using chemically modified probes, we designed a lateral-flow (LF) immunoassay that can be used in combination with RIDA for equipment-free nucleic acid target detection. RIDA is a “probe amplification” assay that uses the single-strand nicking activity of restriction nicking endonucleases to repeatedly cleave synthetic probes hybridizing to the same target sequences. In the RIDA-LF combined assay, chemically labeled probes are covalently conjugated to magnetic microbeads, which is propitious to separate cleaved probes from the reaction solution. The cleaved probes in the solution are then detected with an LF immunoassay. The real-time assay shows that RIDA is able to specifically detect target sequences in 5 to 15 min. The RIDA-LF combined assay can specifically detect nucleic acid targets without sophisticated equipment. In this report, our data suggest that RIDA is a flexible simple assay that could be applied for point-of-care detection. The modified-RIDA described in this report further extends the application of this technology.

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1. Introduction

In the past 2 decades, the mainstream techniques applied for pathogen diagnosis have transited from protein-based techniques to nucleic acid testing assays. Nucleic acid testing assays are not only important for the detection of very small amount of DNA or RNA, but are also very useful to identify organisms that are difficult to culture or ascertain by standard histologic methods. In addition, nucleic acid testing assays are able to identify antiviral resistance and sequence genomes of infectious organisms, which are difficult to achieve through the detection of antigens or antibodies. A number of nucleic acid testing techniques have been developed. In general, these methods fall into 3 categories: i) target amplification systems, ii) probe amplification

systems, and iii) signal amplification systems. Within each category, the methods can be also divided based on whether the reaction requires thermal cycler, such as polymerase chain reaction (PCR) (Mullis et al., 1986; Saiki et al., 1986), or react under isothermal conditions. Isothermal methods, which require no thermal cycler, are superior over PCR because PCR requires a temperature cycling protocol to achieve amplification, thus, imposes instrumentation constraints on the PCR method, which make it more complex and limit the rate of the amplification to the temperature cycling schedule. Among the isothermal methods developed, nucleic acid sequence-based amplification (Kwoh et al., 1989; Kievits et al., 1991; Guatelli et al., 1990; Compton, 1991), transcription-mediated amplification, strand displacement amplification (Walker et al., 1992a, 1992b), and rolling circle amplification (Liu et al., 1996; Lizardi et al., 1998; Fire and Xu, 1995) represent the isothermal target amplification systems; techniques such as invader assays (Olson et al., 2004; Kwiatkowski et al., 1999) represent the isothermal probe amplification systems. These tests can reach extreme sensitivity and considerably reduce the detection time from

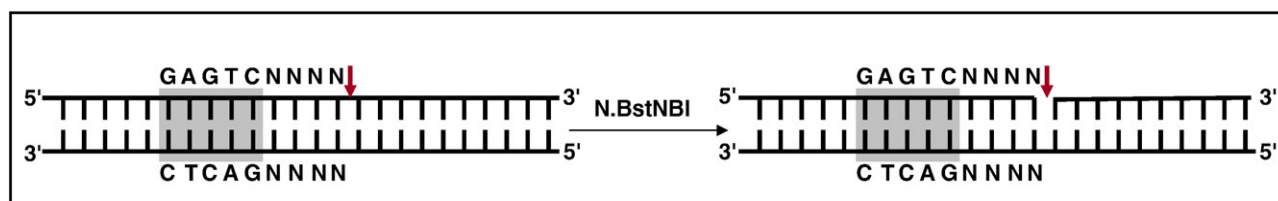


Fig. 1. Schematic diagram of N.BstNBI recognition site and its nicking site. The gray box indicates the double-strand N.BstNBI binding site; red arrow, N. BstNBI nicking site. The recognition sequence of N.BstNBI is 5'-GAGTC-3' and cleaves 4 bp downstream from the 3' end.

days to hours while maintaining the level of specificity required for the diagnostic testing. However, limitations, such as complicated reaction systems, high costs, the need for specialized equipment, and the lack of standardized assays and personnel training, have restricted the widespread use of these assays in the clinical setting (Fortina et al., 2002). Although some molecular diagnostic tests are affordable, the requirements for trained technicians result in higher costs for implementing the assays. Another drawback of nucleic acid testing is that many of these assays are not available in the clinical diagnostic laboratory because of costs related to equipment. Thus, new rapid, accurate, affordable, and simple assays are needed for underdeveloped locations.

A new rapid isothermal nucleic acid detection assay (RIDA) is described here. RIDA is achieved through the binding of a reporting probe (RP) to the target DNA or RNA, followed by the nicking on the RP with the restriction nicking endonuclease. A number of restriction nicking endonucleases have been obtained from nature or through recombinant engineering. These restriction enzymes recognize a specific double-strand binding site and make the cut at only 1 strand (Zheleznaya et al., 2002a, 2002b). N.BstNBI is an enzyme identified from *Bacillus stearothermophilus* (Higgins et al., 2001). It recognizes the sequence of 5'-GAGTC-3' and cleaves 4 bp downstream of the 3' end (Fig. 1). N.BstNBI functions from 37 to 65 °C, with an optimum temperature at around 55 °C. The principle of a basic RIDA is illustrated in Fig. 1. RIDA belongs to a “probe amplification system”, which uses a specific synthetic single-strand DNA as an RP. In a basic RIDA, the RP contains 5'-GAGTC-3'. Under a certain reaction temperature, the RP forms stable double strands with its complementary target sequence and forms the N.BstNBI recognition site. The N.BstNBI enzyme produces a single-strand nick on the RP, which results in the reduction of the melting temperature (T_m) and the subsequent release of the 2 smaller ssDNA probes. When the RP is in excess amount, a new RP will hybridize with the same target and form new double strands, and the new RP could be nicked again. Thus, in RIDA, the same target could be used repeatedly to cleave multiple copies of RPs, which generates large amount of smaller fragments. As a result of RIDA, the existence of the target sequences (DNA or RNA) is indicated through the generation of cleaved RPs.

In this report, for the purpose of proving the principle of RIDA, we use RIDA for the detection of the hemagglutinin

(HA) gene of the influenza virus (H5N1). Furthermore, we describe an integrative rapid nucleic acid strip assay that combines RIDA with the conventional lateral-flow (LF) immunoassay. Using the simple RIDA-LF combined strip assay we developed, HA RNA of H5N1 influenza virus can be specifically detected within 25 min. RIDA can be performed in a nonlaboratory point-of-care (POC) environment and can be used to develop a simple disposable diagnostic device.

2. Materials and methods

2.1. Primers, reverse transcriptase PCR, cloning, and in vitro transcription

Target RNA was amplified using 1-step reverse transcriptase (RT)-PCR kit (Takara Biotechnology [Dalian], Beijing, China) following manufacturer's instructions. The primers for RT-PCR and cloning H5N1-HAF (5'-cgcaagcttggcgaagtgaagaatggagtt-3') and H5N1-HAR (5'-cccggatccgatggtgagagggtgtatgt-3'), were synthesized by Invitrogen Biotechnology, Shanghai. In brief, influenza virus H5N1 total RNA (Huayin Biotech, Beijing, China; an avian isolate from Guangdong, China) was mixed with RT-PCR mixture (10 µL 2× 1-step RT-PCR buffer, Takara ExTaq HS 0.2 U, M-MLV RTase 40 U, RNase Inhibitor 16 U, 4 pmol of H5N1-HAF primer, and 4 pmol of H5N1-HAR primer); the final volume of the reaction was 20 µL. RT-PCR was conducted at 42 °C for 15 min, 95 °C for 2 min, followed by 40 cycles of denaturing at 94 °C for 5 s, annealing at 55 °C for 30 s, and extending at 72 °C for 30 s, and after amplification cycles, an extra extension at 72 °C for 5 min. RT-PCR products were digested with *Bam*HI and *Hind*III (New England BioLabs, Ipswich, MA), and cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) following manufacturers' instructions. The product was renamed pcDNA-H5. T7 MEGascript Kit (Ambion Europe, Cambridgeshire, UK) was used for in vitro transcription.

2.2. Real-time RIDA

H5-RP for real-time RIDA, H5-RP (FAM-5'-GCTGAGTCCCCTTTCTTGACAAT T-3'-TAMRA), was synthesized by Invitrogen Biotechnology. Series dilutions of RNA samples were mixed with the RIDA reaction solution (2 µL of 10× NEB buffer 3, 2 µL of bovine serum albumin [BSA] [0.1%], 1 pmol of H5-RP, N.BstNBI [NEB] 2 U); the

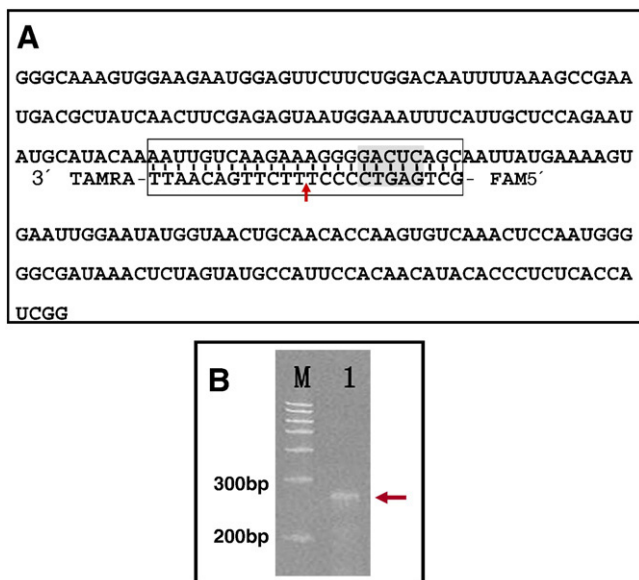


Fig. 2. In vitro transcription of target RNA fragment from H5. (A) The target RNA sequence in vitro transcribed from pcDNA-H5 and the RP used in this assay. The gray box indicates N.BstNBI recognition and binding site; red arrow, N.BstNBI nicking site. (B) Identification of the target RNA sequence in vitro transcribed from pcDNA-H5. Lane M indicates RNA size marker; lane 1, target RNA.

final volume of the reaction was 20 μ L. The mixture was incubated at 55 $^{\circ}$ C, and the progress of the reaction was monitored with an MJ Chormo4 Opticon Monitor System (MJ Research, Waltham, MA) every 30 s.

2.3. Preparation of gold nanoparticles

A chemical reduction method was used to obtain dispersed gold nanoparticles in the solution. One hundred milliliters of 0.01% chloroauric acid solution ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) was heated to boiling with vigorous stirring. Four milliliters of 1% sodium citrate solution was then added to yield colloidal gold particles. The color of the solution changed gradually to violet then to red. After boiling for another 5 min, the mixture was cooled at room temperature and filtered through a 0.45- μ m nylon membrane (Shanghai Minipore Industrial Co., Ltd, Shanghai, China).

2.4. Colloidal gold antibody conjugation and preparation of LF strip

Gold nanoparticle probes were prepared as previously described (Oliver, 1999). Each LF strip was assembled as illustrated in Fig. 5A. The mouse monoclonal antibody (mAb) against sulfamethoxydiazine (SMD) was generated in the laboratory. LF test strip was constructed as described before (Peak et al., 2000) with modification. A gold-labeled SMD antibody probe was jetted onto the untreated glass fiber membrane (Shanghai Minipore Industrial Co., Ltd, Shanghai, China) and dried at room temperature. Rabbit antimouse antibody (GeneTex, San Antonio, TX) and BSA-SMD

antigen (Nanhai Beisha Pharmaceutical, Guangdong, China) in phosphate-buffered saline (PBS) were jet positioned onto nitrocellulose membrane (Sartorius Scientific Instruments, Beijing, China) as the control line (C line) and the test line (T line), respectively. The remaining active sites on the membrane were blocked by incubation with 1% BSA in PBS for 30 min at room temperature. The membrane was washed once with PBS and twice with distilled water, and then dried. The sample pad (Whatman Asia Pacific) and the absorbent pad were used without treatment. The glass fiber membrane, the nitrocellulose membrane, the sample pad, and the absorbent pad were assembled as the strip and attached to the polyvinyl chloride motherboard. The assembled strips were then cut into 3 mm in width.

2.5. H5-RP-SMD and H5-RP-SMD-Magnabead

An assembled H5-RP-SMD-Magnabead was shown in Fig. 4A. The H5-RP-SMD was prepared by covalently conjugating a molecule of SMD (Nanhai Beisha Pharmaceutical) at the 5' end and a molecule of biotin at the 3' end. An H5N1-RP-SMD-Magnabead was prepared through the conjugation of H5-RP-SMD with MagnaBindTM streptavidin beads based on manufacturer's instructions (Pierce, Rockford, IL). In brief, after washing 3 times with PBS, the excess of H5-RPs-SMD was added to wash beads and incubated at room temperature for 30 min. These beads were ready for RIDA-LF reaction after washing with PBS.

2.6. RIDA-LF reaction

H5-RP-SMD-Magnabeads (10 μ L) were mixed with different amounts of H5 RNA (1.50×10^{13} copies, 7.52×10^{12} copies, 3.76×10^{12} copies, 1.88×10^{12} copies), and 2.22×10^{13} copies mock RNA in the RIDA reaction solution in 1.5 mL of microcentrifuge tubes and incubated at 55 $^{\circ}$ C for 20 min after adding the N.BstNBI enzyme (total volume is 30 μ L). The microcentrifuge tubes were then placed on magnetic stands to pellet the beads to solid phase. Reaction products (30 μ L) were then mixed with 30 μ L of water and applied to the sample pad of SMD-LF. Read the result after 5 min.

3. Results

3.1. Identification, cloning, and transcription of an H5N1-specific sequence as a target sequence

The avian influenza virus genome contains 8 segments of single-strand RNAs. To generate enough H5N1-specific RNA for assay development, we identified and cloned an H5N1-specific sequence for in vitro RNA transcription. Compared with the other segments, segment 4 (HA gene), segment 6 (NA gene), and segment 7 (matrix protein gene) are more conservative among different influenza strains. RNA fragments containing 5'-GACUC-3' were identified from HA, NA, and MP gene sequences. After BLAST

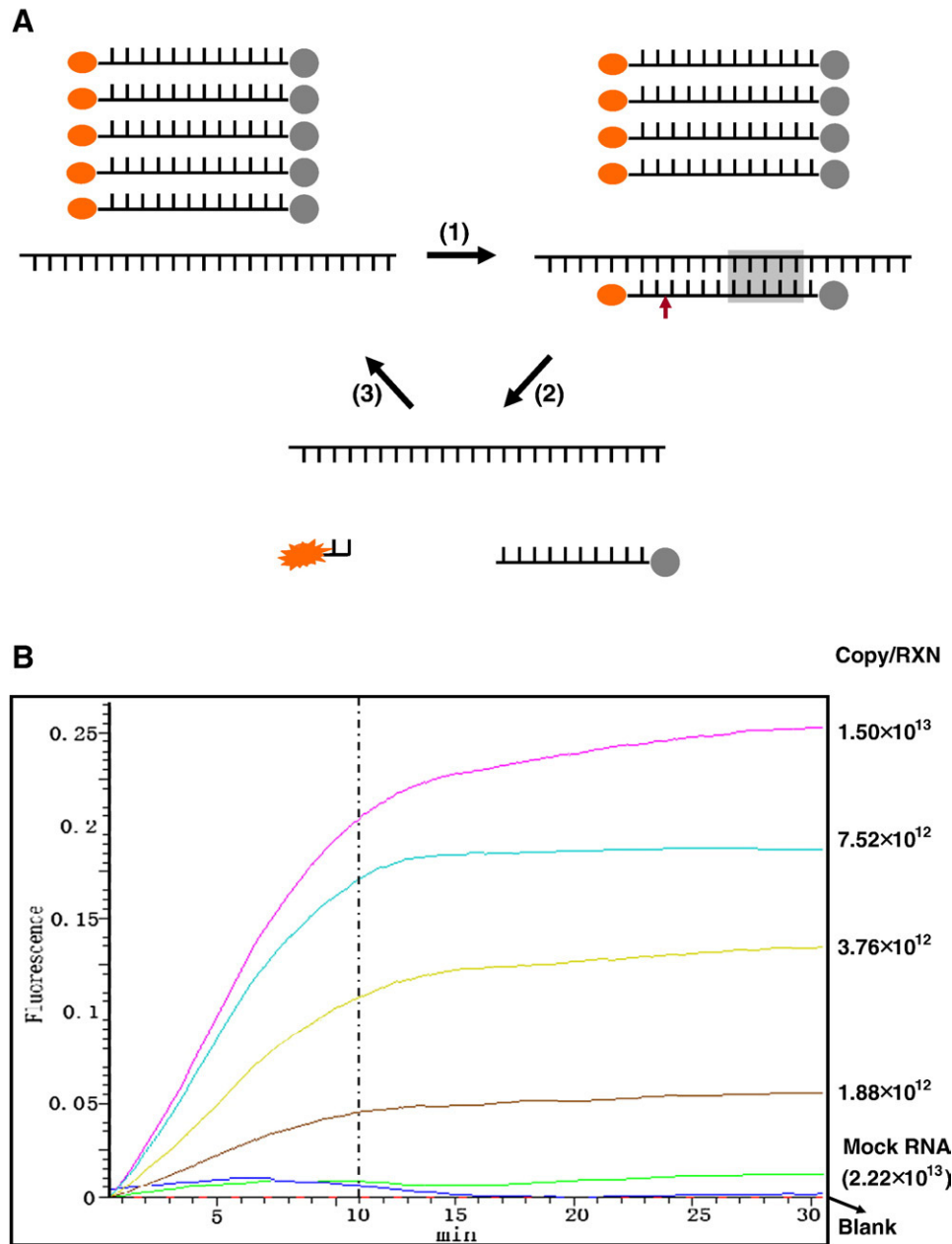


Fig. 3. Real-time RIDA detection of H5 target RNA. (A) Schematic representation of a real-time RIDA. The RP is labeled with a fluorophore at one end (orange) and a quencher at the other end (gray), which allows for real-time monitoring of the progress of RIDA reaction. (1) In the reaction mix, excess amount of RPs are mixed with the nucleic acids extract. Some of these RPs hybridize on the target sequence and form N.BstNBI recognition sites. (2) The hybridized RP is nicked, which results in 2 shorter fragments and falls off from the target sequence under the reaction temperature. (3) A new RP hybridizes on the target sequence from (2) and the reaction (1) repeats. (B) Different amount of H5 target RNA that is in vitro transcribed RNA from pcDNA-H5 is used in the reactions, which are shown as COPY/RXN. Mock RNA, in vitro transcribed RNA from pcDNA3.1(+).

searches, 1 fragment from HA (Fig. 2A) was identified as a unique sequence fragment, which was used as the target sequence. This fragment was further cloned into pcDNA3.1 (+). The product was renamed pcDNA-H5. The pcDNA-H5 was in vitro transcribed under T7 promoter (Fig. 2B).

3.2. Real-time RIDA detection of H5 RNA

The progression of RIDA reactions was monitored on an MJ Chormo4 Opticon Monitor System using software

supplied by the manufacturer. The real-time measurements were made under isothermal conditions at 30-s interval after the temperature of the sample reached 55 °C (Fig. 3A). In this assay, the RP was labeled with a fluorophore at one end and a quencher at the other end. The probe was non-fluorescent because of the close proximity of the fluorophore and the quencher. At 55 °C, this “detection probe” hybridized with the target and forms the double strands containing the N.BstNBI recognition site. The T_m for the

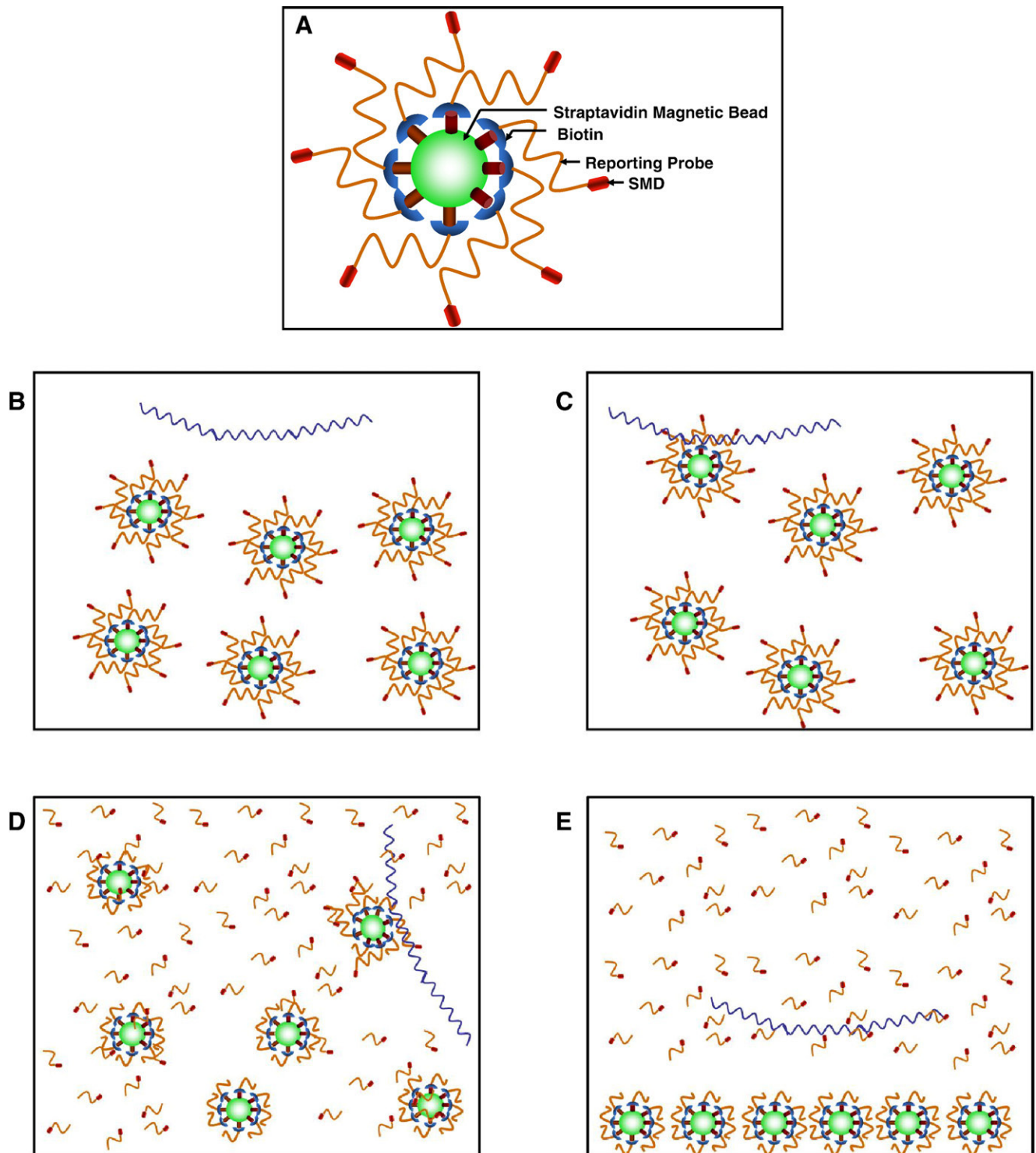


Fig. 4. Schematic diagram of RIDA reaction on magnetic beads. (A) The RP is labeled with biotin (blue) at 3' end and SMD (red) at 5' end. This RP is immobilized on streptavidin magnetic beads. (B) The RP-conjugated magnetic beads are mixed with target RNA (blue plot) in suspension. (C) Target RNA hybridizes on the RP on beads in suspension. (D) RIDA reaction generates cleaved RPs. The SMD-labeled fragments are released from beads into the solution. (E) Magnetic beads are immobilized by applying with magnetic force.

double strands was 63.3 °C. Under the isothermal condition, in the presence of N.BstNBI enzyme, the detection probe was nicked to 2 shorter fragments, with Tms of 45.3 and 36.3 °C. At 55 °C, these 2 short double strands were

unstable, thus, separated from the target RNA; as a result, the fluorescence could be detected in real time by a fluorescence reader. After the separation from the cleaved detection probe, the target RNA molecule became single stranded

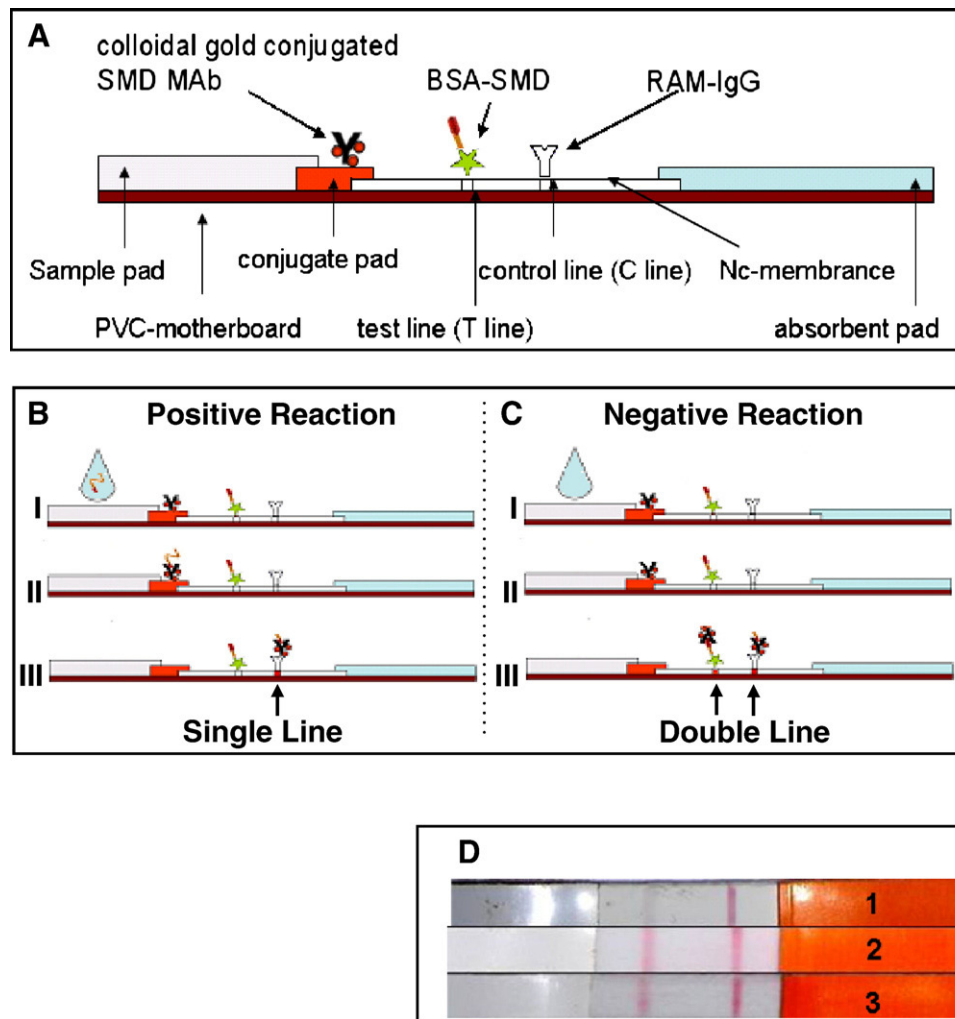


Fig. 5. Schematic diagram of the LF immunoassay to detect SMD released in the solution. (A) Diagram of the strip for the SMD LF immunoassay. (B) Diagrams of a positive SMD LF immunoassay. The colloidal gold-labeled anti-SMD antibody on the conjugate pad binds free SMD in the solution, which loses its capability to bind SMD fixed on the T line. A positive result is indicated by weak or no formation of T line. In this LF immunoassay, the colloidal gold-conjugated anti-SMD-mAb was attached to the conjugate pad. In this case, SMD is released to the solution, and SMD binds to this mAb, which competitively blocks its binding to SMD on the T line (5B-II). Following the flow of liquid, the SMD-mAb (colloidal gold) complex is captured by a secondary antibody on the C line (5B-III). Therefore, a single line at the position of the C line indicates a positive result. (C) Diagrams of a negative SMD LF immunoassay. The colloidal gold-labeled anti-SMD antibody on the conjugate pad binds SMD fixed on the T line. A negative result is indicated by strong formation of T line. In this case, there is no SMD released to the solution, and the SMD-mAb (colloidal gold) complex binds SMD-BSA on the T line (5C-II), which forms the T line. The excess amount of mAb will cross the T line and be captured on the C line. Thus, the formation of both the T line and C line is an indication of a negative result (Fig. 5C-III). (D) LF detection of SMD from RIDA reaction on magnetic beads. For each strip, 7.8×10^{10} copies of H5 RNA fragment (strip 1), no H5 RNA fragment (strip 2), and RNA in vitro transcribed from pcDNA-H5 (strip 3) were used.

again, which was able to hybridize with another full-length detection probe, and repeated the above reaction. As shown in Fig. 3B, the intensity of fluorescence was corresponding to the amount of target RNA in the reaction. Within the 1st 10 min of the reaction, the intensity of fluorescence increased linearly. As an indication of the specificity of the reaction, RNA from pcDNA3.1 (+) transcription gave no increase of signal. These results suggested that RIDA could detect the H5 target sequence specifically. In addition, RIDA progressed linearly within the 1st 10 min of reaction, which could be applied for the quantitative estimation of the amount of the target sequence.

3.3. RIDA-LF assay

To further simplify the procedure of RIDA, we devised a new format of RIDA-LF assay including the following 2 sequential reactions: First, we performed a RIDA reaction on magnetic beads to generate cleaved RP fragments (Fig. 4). In this RIDA reaction, each molecule of RP was covalently conjugated to a molecule of biotin at the 3' end and a molecule of SMD at the 5' end. This RP was immobilized on a streptavidin magnetic bead (Fig. 4A). Without applying magnetic force, these beads were mixed with H5 RNA in suspension at 55 °C (Fig. 4B), which allowed efficient hybridization between the target RNA and RPs

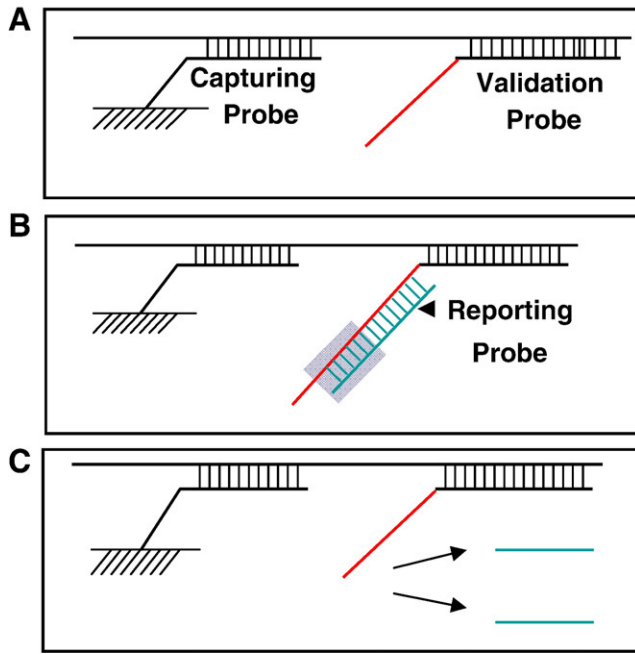


Fig. 6. A modified RIDA to detect target sequences without specific sequence requirements. (A) The capturing probe on solid phase captures the target sequence. The validation probe binds to a remote site of the same target. (B) The validation probe binds to the RP and forms the nicking enzyme recognition site. (C) RIDA reaction.

(Fig. 4C). In the presence of N.BstNBI, the RP was cleaved into 2 fragments, which resulted in the releasing of the 5' ends (with SMD conjugated) to the solution (Fig. 4D). When magnetic force was applied to this mixture, the magnetic beads would be pelleted to solid phase, which cleaved SMD in the solution (Fig. 4E).

Subsequently, we utilized a standard LF immunoassay to detect SMD released in the solution (Fig. 5A–C). In this LF immunoassay, the colloidal gold-conjugated anti-SMD mAb was attached to the conjugate pad. Thus, when SMD was

released to the solution, SMD would bind to this mAb, which would competitively block its binding to SMD on the T line (Fig. 5A, B-II). Following the flow of liquid, the SMD–mAb (colloidal gold) complex would be captured by a secondary antibody on the C line (Fig. 5B-III). Therefore, a single line at the position of the C line indicated a positive result. On the contrary, if there was no SMD released in the solution from the RIDA reaction, the SMD–mAb (colloidal gold) complex would bind SMD–BSA on the T line (Fig. 5C-II), which formed the T line. The excess amount of mAb would cross the T line and be captured on the C line. Thus, the formation of both the T line and C line was an indication of a negative result (Fig. 5C-III).

We used the RIDA-LF assay to detect the H5 target RNA. After magnet immobilization, 30 μ L of the reaction solution was mixed with 30 μ L of water and added to the sample pad. The LF immunoassay result was shown in Fig. 5D. When H5 RNA fragment was included in the reaction, a single C line was formed in 5 min (Fig. 5D, strip 1). When there was no H5 RNA added (Fig. 5D, strip 2), or when control RNA was added (Fig. 5D, strip 3), both the T line and C line form.

4. Discussion

In this report, utilizing the special property of restriction endonuclease nicking enzymes, we developed RIDA, a novel rapid nucleic acid detection assay. Furthermore, we provided a simple solution to adapt a standard LF immunoassay for the detection of nucleic acids probe fragments generated by RIDA.

In the past, there are several reports of probe amplification assays. Most of these assays utilize cycling probe technology (CPT) (Bekkaoui et al., 1996, 1999; Bhatt et al., 1999; Modrusan et al., 1998; Fong et al., 2000; Modrusan et al., 2000; Warnon et al., 2000; Tang et al., 2002; Harvey et al., 2004). CPT utilizes a chimeric DNA–RNA–DNA

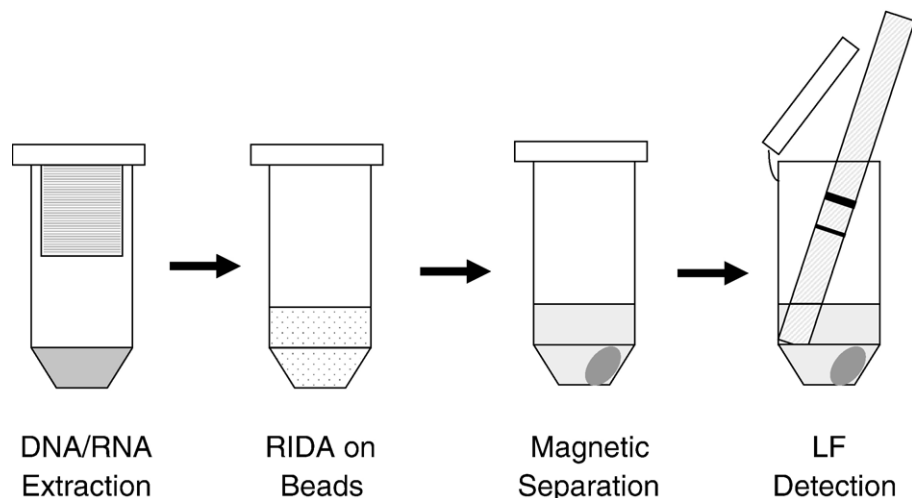


Fig. 7. Schematic diagram of 1 possible RIDA-LF reaction process.

probe, which is cleaved by RNaseH when hybridized with its complementary target DNA. CPT and RIDA achieve the purpose of detection through employing the same target sequence repeatedly during the reaction, which eliminate the possibility of introducing errors during target amplification steps, a concern for PCR assays. Compared with CPT, RIDA is more universal and flexible, mainly due to the replacement of RNaseH with nicking enzymes. First, RIDA could be applied for the detection of both DNA and RNA, separately or together; on the contrary, CPT can only be used to detect DNA targets. Second, RIDA uses a conventional DNA-oligo as the probe, which is much simpler to design and produce than CPT, which required a DNA–RNA–DNA hybrid probe. Third, RIDA is more flexible and multipliable. With differently labeled RPs, RIDA can be used to detect multiple DNA and RNA targets simultaneously. Furthermore, the real-time RIDA and the RIDA-LF results suggest that RIDA could be used for rapid, accurate, affordable, and simple detection. With excess of RPs, RIDA detection could be achieved in 15 min in the same tube. Because nontarget RNA could not form the recognition site for the nicking enzyme, excess heterologous nucleic acid dose not reduce the sensitivity of the amplification reaction (Fig. 4) even at 10-fold higher amount (Fig. 3). Our results also indicate that excess heterologous nucleic acid would not react with the strip (Fig. 5B, C). In addition, RIDA is semiquantitative; this is indicated by our florescence-based RIDA (Fig. 3B) and RIDA-LF (Fig. 5).

LF type of assay is considered the best POC assay currently available, mainly because of its simplicity, speed, and very limited hardware requirements. In the past, several methods were developed to use LF for the detection of nucleic acid (Seal et al., 2006; Wang et al., 2006; Malamud et al., 2005). Most of these methods rely on target amplification and detection of nucleic acid products directly, thus, requiring significant changes of the classic LF setup, including using special membranes for chromatography. With CPT, Fong et al. (2000) applied a dual fluorescein and biotin-labeled oligo probe to detect single-stranded amplicon. However, the utility of such standard fluorophores is limited by high background fluorescence, the need for a complex reader, and the number of spectrally diverse fluorescein available for multiplexing. In this study, we decided to label RPs with SMD to avoid the limitation of fluorescein labeling. In addition, SMD is small; thus, its interference to RIDA is limited during the reaction, plus LF immunoassays for SMD detection are already available. Using an SMD LF immunoassay as an example, our data presented here suggest that LF immunoassays are fully adaptable for combined RIDA-LF assay. This finding further extends the possible applications of RIDA.

Although the RIDA assay we described here requires the presence of a nicking enzyme recognition site within the target sequence, a modified RIDA could be used for the detection of targets with no specific sequence requirements. As shown in Fig. 6, this modified RIDA includes a “capture

probe” to attach the target sequence to a solid phase, which allows the separation of the targets from the other nucleic acids in the sample mixture. The 2nd probe (“validation probe”) has 2 parts: the sequence that is complementary to the target and the sequence with a restriction enzyme recognition site. The validation probe attaches to the target that is linked to solid phase through the capture probe. A 3rd probe (RP) then hybridizes with part of the detection probe, which can be nicked by nicking enzyme. Development of microarrays with capture probes for different targets is probably a logical application of this modified RIDA. Such flexibility of RIDA warrants easy combinations with the other isothermal technologies, in particular, methods like SMART (Wharam et al., 2001), which is capable of detecting both DNA and RNA.

Compared with the other double-strand cutting restriction enzymes, nicking enzymes are a class of newly discovered endonucleases. Although most of the nicking enzymes commercially available are generated through recombinant technologies, there are nicking enzymes existing in nature microorganisms. N.BstNBI is a type II restriction nicking enzyme that was isolated from thermophilic bacterium *B. stearothermophilus* (Morgan et al., 2000). Two major properties of N.BstNBI lead to the selection of this enzyme in our system. First, we found that N.BstNBI could be used on both DNA (Higgins et al., 2001) and DNA–RNA hybrid as its substrate, thus, enabling the system to detect both DNA and RNA targets. Second, N.BstNBI has an optimal reaction temperature of 55 °C (Morgan et al., 2000), which makes the selection of an appropriate RP easy. However, N.BstNBI is still not an ideal enzyme for RIDA reaction because it is not stable above 65 °C (Morgan et al., 2000), thus, limiting the application of this system at higher temperature. In the future, with more nicking enzymes discovered/generated, a better enzyme can be used to carry RIDA reaction more efficiently.

In conclusion, we present here a rapid and simple approach for nucleic acids detection, RIDA, and RIDA-LF. Our data suggest that RIDA is flexible and multiflexible. In combination with a nucleic acid extraction process, an equipment-free RIDA-LF could be performed as illustrated in Fig. 7. Adaptations of this method can be applied in broad applications in molecular biology.

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